

CXLIV. SOME OBSERVATIONS ON THE EXTRACTION AND ESTIMATION OF LIPOCHROMES FROM ANIMAL AND PLANT TISSUES.

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DURING some recent investigations of the lipochrome content of various animal and plant tissues, certain observations made it seem desirable to examine some of the generally adopted methods of extraction, separation and estimation rather carefully. The results obtained may prove of use to other investigators in the same field.

It was hoped at first to adapt the simple method of grinding up the tissue with anhydrous sodium sulphate and extracting with light petroleum to a quantitative basis. The procedure adopted was as follows. The tissue to be examined was quickly cut into small pieces, a little ether was added to stop enzyme action, about 0.5 g. solid potash to decompose chlorophyll where present and the whole rubbed up with sand and anhydrous sodium sulphate. The dry mass was then extracted with light petroleum (B.P. below 40°) in a Soxhlet extractor for 2-3 days until no more pigment was taken out. The coloured extract was then evaporated down to a small bulk in a current of nitrogen, and submitted to Kraus' phase test. It was transferred to a stoppered measuring cylinder, an equal volume of 90 % methyl alcohol added, the mixture well shaken and the resulting volumes of the two liquids noted. (The total volume does not alter.) The carotene is dissolved almost completely in the upper layer of light petroleum and the xanthophyll in the lower layer of 90 % methyl alcohol. By separating these two liquids and further extracting each with a fresh portion of the other solvent, a very little pigment can be dissolved out of each phase. The second extracts so obtained appear to be about equally concentrated but in any case so dilute as to be unmeasurable by the only means (the Hellige colorimeter) at our disposal. Hence in subsequent operations the second extraction of each phase was not carried out. The total carotene and xanthophyll were calculated from the noted volumes and concentrations.

The only means at present available of estimating carotene or xanthophyll quantitatively is by comparing the given solution colorimetrically with one of known strength; or, as solutions of carotene and xanthophyll are unstable,

Willstätter proposed comparison with a solution of potassium dichromate which will keep indefinitely. A convenient strength to use is one of 0.2 % in water, but there is no straight line relation between the concentration of lipochrome solution and depth of colour of the dichromate solution. The very few data given by Willstätter and Stoll [1913] have been plotted graphically by Palmer [1922] for use with a Kober, Duboscq or Wolff colorimeter. As this is not directly applicable to readings obtained by the Hellige colorimeter, a fresh curve was made for this apparatus. The dichromate solution (0.2 %) is contained in a movable wedge of glass, the solution to be estimated in a stationary vessel whose front and back faces slope at the same angle as those of the movable wedge. These two vessels are so arranged in the apparatus that the colours are viewed side by side and the dichromate wedge can be raised until it matches the solution to be estimated. By starting with a strong solution of freshly crystallised carotene of known strength (about 2.5 mg. in 100 cc. light petroleum is suitable), making various dilutions and matching the dichromate solution to each one, a curve may be obtained by means of which any depth of carotene solution may be interpreted as milligrams of carotene per 100 cc. of solvent. The curve obtained for carotene is given in Fig. 1, p. 1121. It was confirmed by another worker (V. B. R.) in this laboratory and is practically identical with that obtained from a different sample of crystalline carotene.

Three preparations of xanthophyll crystals were made from dried nettle leaves by Jörgensen and Stiles' [1917] modification of the method used by Willstätter and Mieg [1907]. They served for spectrum and chromatographic analysis, but on attempting to recrystallise for an estimation curve, a brown oily liquid formed each time and fresh crystals could not be obtained. Hence, where necessary, the writer used the curve for xanthophyll very kindly lent to her by Dr O. Rosenheim.

The method of extraction of lipochromes detailed above gave such widely different results in total and relative amounts of carotene and xanthophyll extracted from different samples of the same material that certain points in the method were investigated.

In the first place, by working with a known amount of carotene in light petroleum solution, practically no lipochrome was recovered, and a similar loss was experienced in using sand and no anhydrous sodium sulphate. Potash seemed to destroy the whole of the carotene.

Adsorption on sodium sulphate was estimated by using known amounts of lipochrome and of the salt and quickly extracting in the usual way. The loss is shown in Table I, and is apparently proportional to the weight of sulphate used.

The variability in the ratio, carotene : xanthophyll, was investigated by extracting 50 g. etiolated wheat shoots prepared by the method already described and estimating the carotene and xanthophyll in successive fractions of the extract. From Table II it will be seen that the carotene is apparently

extracted earlier in the operation than the xanthophyll, which would account for a difference in the ratio of these two substances if the extraction had not been completed.

Table I.

	Anhydrous Na ₂ SO ₄ g.	Vol. of sol. cc.	mg. carotene in this vol. of sol.	mg. carotene recovered	Total loss	% loss
A	5	5	0.10625	0.0875	0.01875	17.6
B	10	5	0.10625	0.0712	0.03505	33.0

Repeated with a weaker solution.

C	25	25	0.06	0.0475	0.0125	20.8
D	50	25	0.06	0.035	0.025	41.7

Table II.

	Estimated at end of	mg. carotene obtained	mg. xanthophyll obtained	Ratio C:X
1st fraction of extract	24 hrs	0.0036	0.0112	0.32
2nd " "	28 "	0.0028	0.0133	0.21
3rd " "	72 "	0.0023	0.0132	0.17
4th " "	120 "	0.00167	0.017	0.098

Hence the method of grinding up with potash and anhydrous sodium sulphate and extraction in a Soxhlet was discarded for quantitative work.

Saponification with *alcoholic* potash and subsequent extraction does not give quantitative results, as much yellow resinous material is formed and extracted by the light petroleum. Saponification with *aqueous* potash however gives satisfactory results, though in this case it is necessary to add about 20 cc. alcohol to the product before extraction, otherwise no lipochrome at all is extracted. The whole process must also be carried out as nearly as possible in an atmosphere of nitrogen. To compare the influence of different strengths of potash solution, 10 cc. of a light petroleum solution of carotene (containing 0.115 mg. carotene) together with 5 g. dried egg albumin (to imitate somewhat the conditions of an ordinary saponification) were saponified with 200 cc. of potash solutions of 50, 25, 18.75, 12.5, 6.25, 2.5 % respectively. The extracts were made up to 10 cc. the original volume of lipochrome solution. The Hellige reading of the original solution was 55.6, those of the extracts 56.4, 55.7, 55.6, 55.5, 55.4, 55.5 respectively (each an average of nine readings). The first indicates a loss of 0.003 mg. in 0.115 mg. carotene and was obtained with unnecessarily strong potash. The rest may be regarded as quantitative results.

Tswett's Chromatographic Method of Analysis of Lipochromes.

Tswett [1906, 2] claims to have separated four different xanthophylls from the pigments of green leaves by his chromatographic analysis. He names them α , α' , α'' , β respectively and gives their spectra as:

	Band I	Band II	Band III
Xanthophyll α (nearest the base)	485-470	455-440	—
Xanthophyll α'	Slightly shifted towards the violet		
" α''	475-462	" 445-430	" —
" β			

From his wide investigations of the adsorptive powers of different substances (some 30–40) for these pigments and for other substances, and also from the fact that his four xanthophylls give different spectra, Tswett [1906, 1] assumes that they are definitely different substances. He appears, however, to have examined the lipochromes of green or yellowing leaves only, by this method. Hence it seemed desirable to examine other tissues by this method as they might give a greater or smaller number of xanthophylls or different lipochromes altogether.

The perianths of the polyanthus narcissus were first examined and were found to be such a convenient and rich source of the lipochromes that this material was used for testing several points in the principle of the chromatographic method. Ten single flowers gave a solution strong enough for the purposes of a chromatograph. The tubes of the perianths were discarded as they were somewhat green. The tissue was ground up with anhydrous sodium sulphate and quickly extracted (but never completely so) with cold light petroleum. The chromatograph was made at once and washed thoroughly with the pure solvent. The column was removed, the layers separated and extracted with ether. The solutions gave the following spectra:

	Band I	Band II	Band III	Substance presumably
Filtrate	489–469	454–439	425 to end	Carotene
Layer (a) near base	482–467	451–436	425 "	Xanthophyll α
" (b)	480–465	449–434	422 "	" α'
" (c)	478–465	449–434	221 "	" α''
" (d) near top	476–464	447–431	420 "	" β
Size of slit of spectro- scope (mm.)	0.08 0.08	0.08 0.095	0.12	

In each case the spectrum was read several times, an average made and the result expressed to the nearest $\mu\mu$.

This experiment has been repeated several times, each time with similar results. Incidentally, it was found that in using a strong solution of the lipochromes, xanthophyll α was easily retained in the chalk but that the other three xanthophylls were not very widely separated. On the other hand, by using a weak solution, the xanthophylls α' , α'' and β were more widely separated but xanthophyll α was washed out into the flask together with, or just behind, the carotene. By passing this mixture of xanthophyll α and carotene through a fresh chalk column in more concentrated solution, they were conveniently separated. Also, after thorough washing of a chromatograph with the pure solvent (light petroleum was used throughout in these experiments) any one well-defined layer could be extracted with ether, evaporated in a current of nitrogen, taken up in light petroleum and another chromatograph attempted with it. But in each case, only one layer was obtained and the filtrate was colourless. Furthermore, on combining the extracted, evaporated and redissolved pigment from any one layer with the filtrate from the original chromatograph, only one layer was formed in a fresh chromatograph and the fresh filtrate was coloured.

Thus it would appear that the four xanthophylls really are different substances, separated as Tswett suggested by their different adsorption affinities for the chalk.

The lipochromes of a very deeply-pigmented daffodil, "King Alfred," have also been examined in this way, and four distinct layers obtained. Their spectra were:

	Band I	Band II	Band III
Xanthophyll α	482-467	449-436	418? to end
" α'	478-465	448-434	417 " { Possibly the same
" α''	478-464	446-433	416 " { xanthophyll
" β''	476-460	444-432	418? "
Size of slit of spectroscope (mm.)	0.08 0.08	0.08 0.095	0.12

This material also was used to demonstrate the fact that the lipochrome of a single layer from one chromatograph gave only a single layer on passing through a fresh chalk column and that the lipochrome of any one layer combined with the filtrate gave only one layer and a coloured filtrate on attempting a fresh chromatograph.

Three occurrences of a very curious phenomenon have been observed during the making of some forty or fifty chromatographs. Practically always the four xanthophylls may be depended upon to take up their positions as indicated, but three times xanthophyll β , identified by its spectrum, has taken up a position below xanthophyll α'' , and once this was seen to happen during the washing when it had already been stationary for some little time at the top of the column. Whether a different sample of light petroleum was used at this point is not recorded.

Crystalline xanthophyll, prepared by the method of Jörgensen and Stiles and containing some sterol, dissolved readily in light petroleum. Its solution gave the spectrum 482-465, 450-435, 425 to end. On attempting a chromatograph, only one layer was obtained which widened slightly on prolonged washing. This was dissolved in ether and the solution gave a spectrum 482-466, 450-434.5, 425-end. On repeating with a weaker solution of crystalline xanthophyll, the following spectra were obtained:

	Band I	Band II	Band III
Before chromatographic analysis	482-467	451-435.5	424.5 to end
After " "	482-467	452-436	425 " "

A Separation of Lycopin and Carotene.

While investigating various sources of lipochromes for Tswett's four xanthophylls, the corollas of the ray florets of the African marigold (*Tagetes erecta*) were examined. A light petroleum extract of this tissue was passed through a chalk column in an attempt to get a chromatograph. The coloured filtrate first obtained was pinkish-yellow and a second and third fraction of the filtrate were a similar colour but the fourth was distinctly more greenish-yellow than the previous ones. This suggested the possibility that some lipochrome might have passed through the column more easily than the carotene. Spectrum analysis of the fractions gave the following results:

		Band I	Band II	Band III	Band IV	Lipochrome
1st	fraction of filtrate	500-483	467-453	428-419	413 to end	Some lipochrome other than carotene
2nd	" "	490-479	452-440	428 to end	—	Carotene
3rd	" "	491-481	452-440	428 "	—	"
4th	" "	*	453-441	428 "	—	"

* Absent even on concentrating considerably.

Various observers report the following readings of spectra of lipochromes:

Lipochrome	Band I	Band II	Band III	Solvent	Observer
Lycopin	510-499	480-468	440 to end	Alcohol	Willstätter and Escher (1910)
Carotene	492-478	459-446	—	"	Willstätter and Stoll (1913)
"	490-475	455-445	430-418	"	Kohl (1902)
"	490-475	455-445	430-418	Ether	"
"	492-475	460-445	—	Light pet.	Tswett (1911)
"	488-470	456-438	—	Alcohol	Willstätter and Mieg (1907)

The lipochrome other than carotene observed in the filtrate from the extract of pigments from the African marigolds does not appear to be lycopin. It gave the typical colour reactions for lipochromes with concentrated sulphuric and nitric acids but no further means were taken to determine its nature. The interest of the observation lay in the suggestion of a possible means of separation of two lipochromes such as lycopin and carotene.

This experiment was repeated with a light petroleum extract of tomatoes dried with anhydrous sodium sulphate. The chief lipochrome of this fruit is lycopin. On passing through a chalk column, the first fraction of the filtrate was a pure yellow, the second fraction taken was slightly pink, the third distinctly pink, and also the fourth. Left in the chalk were two sharp yellow bands near the top of the column, and a wide very diffuse pink part which would probably have passed out completely on further washing. The spectra of these parts were:

	Band I	Band II	Band III	Lipochrome
1st fraction of filtrate	487-471	453-440	424 to end	Carotene
2nd " "	487-471	453-440	427 "	"
3rd " "	507-490	476-457	443-433	Lycopin
4th " "	507-491	477-458	444-432	"
Lowest part of column	508-492	478-459	444-432	"
Middle " "	508-493	479-461	Unreadable	"
Lower yellow zone	482-466	453-442	430 to end	Xanthophyll α
Upper " "	476-462	448 to end	Unreadable	" β

To investigate this further as a possible means of separation of lycopin and carotene, solutions in light petroleum of the substances, each twice recrystallised, were made and mixed in such proportions that the spectrum analysis gave:

	Band I	Band II	Band III
Pure lycopin solution	509-492	479-461	447-435
Pure carotene solution	489-471	456-441	428 to end
Mixture of the above—dilute	489-462	454-436	427 "
Mixture of the above on concentrating	508-492	Rest indistinguishable	

This solution was then passed through a chalk column with suction, as if for a Tswett's chromatograph. The filtrate became coloured before the washing with the pure solvent was begun. This point may be of importance in view of

a later experiment. The filtrate was removed at very short intervals, about 2 cc. being collected each time. The fractions were then examined spectroscopically, being diluted where necessary so that they were of comparable strength.

Fraction of filtrate	Band I	Band II	Band III	Lipochrome
I*	489-473	455-440	425 to end	Carotene
II	489-470	454-438	426 "	"
III	489-470	455-438	426 "	"
IV	489-467	455-440	427 "	"
V	489-468	454-438	425 "	"
VI	489-468	455-439	427 "	"
VII	489-470	455-440	429 "	"
VIII	Very faint trace	487-462	448-437 } 426- }	Carotene + lycopin
IX	509-491	477.5-460	446-436	Lycopin
X	508-493	480-462	447-435	"
XI	509-491	478-461	447-433	"
XII	509-492	478-460	447-434	"

* Very weak solution.

Fractions I-VII were then combined and gave the spectrum:

489-468, 455-438, 428 to end,

but no trace of the first lycopin band even on concentrating so much that the carotene bands could not be read. This solution was filtered through a fresh chalk column and gave the following fractions:

Fraction of filtrate	Band I	Band II	Band III	Lipochrome
I	488-470	455-440	429 to end	Carotene
II	489-468	455-438	427 "	"
III	489-468	454-439	428 "	"
IV	489-468	454-441	429 "	"
V	510-491	478-462	449-435	Lycopin

Fractions IX-XII of the first filtration were then combined and gave 509-490, 477-463, 449-436 which on concentrating gave 510-492, 479-460, 450-436, so that no evidence of the presence of carotene was found by this means. It was then filtered through a fresh chalk column, and five fractions of the filtrate were taken.

Fraction of filtrate	Band I	Band II	Band III	Lipochrome
I	489-471	456-439	426 to end	Carotene
II	488-468	453-440	427 "	"
III	509-491	477-461	447-434	Lycopin
IV	509-490	477-459	447-433	"
V	489-470	455-440	430 to end	Carotene

In this experiment it should be noted that washing with the pure solvent had begun long before the coloured solution had begun to emerge from the tube. The possible significance of this point will be taken into consideration in further work which is contemplated, and may serve as an indication for an explanation of the above observations.

With the exception of the last isolated observation, it had always been rather difficult to remove the last traces of lycopin from the chalk column, though prolonged washing had achieved it. Its removal by ether was immediate and apparently complete.

Fractional filtration, of which an example has been described above, suggests a possible method of separation of closely allied substances, if the adsorbent suitable to the particular case can be found.

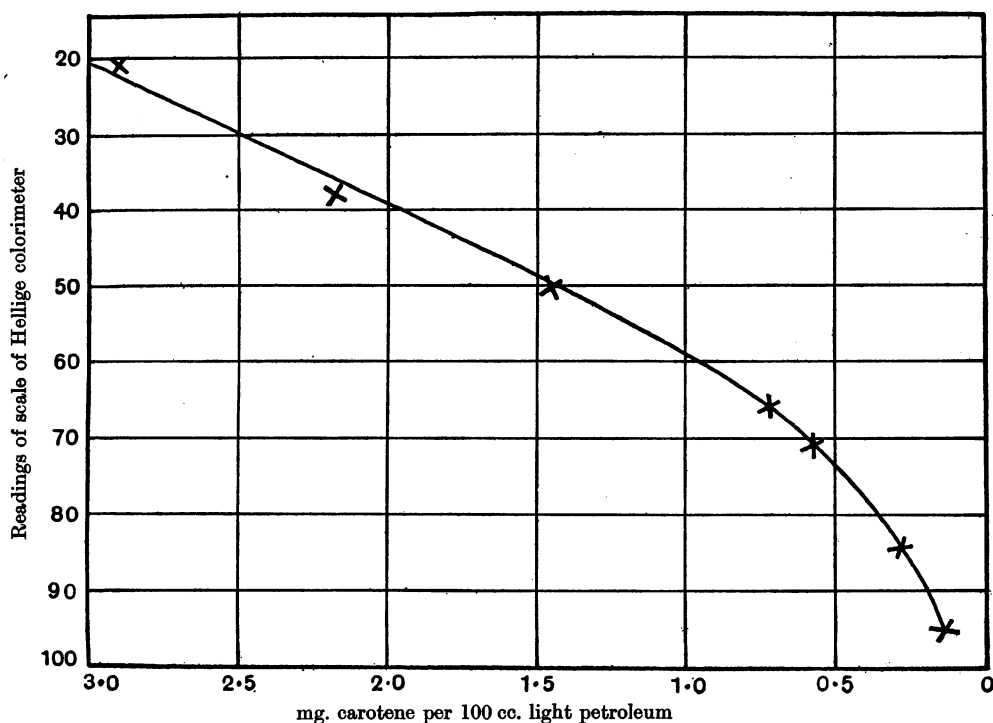


Fig. 1

SUMMARY.

1. Several points in generally adopted methods of extraction of lipochromes have been examined and the results show these methods to be unsuitable for quantitative estimations of the pigments.

2. The only method of extraction giving quantitative results was saponification with aqueous potash of strength not greater than 20 %. Each step of the process should be carried out in an atmosphere of nitrogen as far as possible.

3. A curve for use with a Hellige colorimeter is given for the estimation of carotene.

4. Tswett's chromatographic method of analysis of lipochromes has been examined on many points. It appears to support his theory of the existence of four xanthophylls.

5. Fractional filtration through a chalk column has been found to be suitable for separation of carotene and lycopin from a mixture of the two substances. It may prove useful for the separation of other closely related substances.

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